

# Flavopiridol Induces Apoptosis *via* Mitochondrial Pathway in B16F10 Murine Melanoma Cells and a Subcutaneous Melanoma Tumor Model

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**ABSTRACT** Flavopiridol is a cyclin-dependent kinase (CDK) inhibitor that promotes cell cycle arrest. We aimed to examine the anti-proliferative effects of the flavopiridol and oxaliplatin combination on p16INK4A deficient melanoma cells B16F10 and also its apoptotic effects on a subcutaneously injected B16F10 allograft melanoma tumor model. Flavopiridol and oxaliplatin treated B16F10 cell viability was determined by MTT assay. C57BL6 mice were injected with B16F10 cells and treated with flavopiridol after tumor implantation. BRAF and BCL2L1 mRNA expression levels were measured using reverse transcription-polymerase chain reaction (RT-PCR). Caspase 9 and caspase 3/7 activity were determined by activity assay kits. Proliferating cell nuclear antigen (PCNA) and B-cell lymphoma 2 (BCL-2) protein expression levels were analyzed immunohistochemically. Flavopiridol and oxaliplatin decreased cell death. Flavopiridol enhanced caspase 3/7 and caspase 9 activities *in vitro* and *in vivo* in a dose dependent manner *via* the mitochondrial apoptotic pathway. Even though there was a significant increase in Bcl-2 staining, PCNA staining was decreased in flavopiridol-administered mice. Decreased PCNA expression showed antiproliferative effects of flavopiridol which might be the result of cell-cycle arrest. Flavopiridol can be used as a cell cycle inhibitor, which induced apoptosis through the mitochondrial pathway, independently from BCL2 in B16F10 cells and B16F10 injected C57BL6 allografts.

**KEY WORDS:** malignant melanoma, flavopiridol, apoptosis, proliferation

## INTRODUCTION

Malignant melanoma is a neoplasm of melanocytes and is considered a serious health problem due to both annual incidence and death rates (1). Malignant melanoma can develop in any tissue involving

melanocytes including the skin, eye, and mucosal epithelium of the internal ear. Patients with malignant melanoma who have high risk of developing metastatic disease may benefit from adjuvant therapy.

There have been some agents developed to inhibit specific mutations in malignant melanoma cells. Understanding the role of these mutations and activation mechanisms has led to the identification of survival pathways and resistance mechanisms (2).

BRAF is a member of the RAF kinase family of the serine/threonine kinases in the ERK/MAPK pathway. BRAF gene activating mutations (V600E and V600K) are found in approximately 50% of melanomas (3). The constitutive activation of BRAF kinase enhances the RAS–RAF–MEK–ERK pathway and thereby induces melanoma cell survival and proliferation. Vemurafenib is a mutant BRAF inhibitor drug that has been approved by the FDA (4). After vemurafenib, trametinib (5), a MEK inhibitor and dabrafenib (6), a BRAF inhibitor, have been announced for use in treatment of malignant melanoma. KIT mutations have been another molecular change identified in many melanomas. However, KIT inhibitors such as imatinib have not been useful in patients with activating mutations of the c-KIT gene (7).

Almost 10% of patients with malignant melanoma had a family history of the disease, which is linked to a genetic cause on the short arm of chromosome 9. The deleted locus on chromosome 9 hosts the CDKN2A gene, and the germ-line mutations of this gene has been associated with familial malignant melanomas (8,9). The CDKN2A locus has two overlapping genes, p14ARF and p16INK4a, which are entirely different tumor suppressor proteins (10,11). While p16INK4a regulates RB activation, p14ARF regulates the p53 pathway. P16INK4a binds and inhibits the CyclinD/CDK4/6 complex and thereby prevents RB phosphorylation and causes G1/S cell cycle arrest (12). MDM2 functions as a negative regulator of the cell cycle by ubiquitinating p53, which is a well-known tumor suppressor protein. p14ARF inactivates MDM2 and thus activates p53 (13). The loss of p16INK4a and/or p14ARF directly affects cell cycle control (14).

Understanding cell cycle regulation and cell death will be beneficial for identifying the pathogenesis and the appropriate treatment of malignant melanoma. Programmed cell death, apoptosis, represents a complex signaling mechanism consisting of several intracellular and extracellular pathways. Binding of ligands to specific death receptors triggers the extracellular pathway. This receptor-ligand interaction is followed by intracellular caspase cascade. The initiator caspase 8 activates caspase 3 and 7 that induces endonucleases, resulting in DNA fragmentation (15).

On the other hand, the intrinsic or mitochondrial pathway is triggered by the release of cytochrome-c from mitochondria in response to several intracellular disorganizations such as DNA damage, loss of

mitochondrial transmembrane potential, increased oxidative stress, serum starvation, and activation of oncogenes (16). The mitochondrial translocation of pro-apoptotic Bcl-2 family members such as BAX, BID, and BAK proteins assists the release of cytochrome-c (17,18). When cytochrome-c is released into cytosol it forms an oligomeric complex with APAF-1 (apoptosome) (19,20). When the apoptosome complex is formed, it recruits the initiator pro-caspase 9. Activated caspase 9 induces caspase 3 and 7, which lead to DNA fragmentation (21).

The Bcl-2 family of proteins has both pro- and anti-apoptotic characteristics. While Bcl-2, Bcl-XL, Mcl-1, Bcl-W, and Bcl-2L10 proteins contain four BH domains and prevent cell apoptosis, Bim, Bid, Bad, Bik, Puma, and Noxa proteins induce apoptosis (22). Alternative splicing of Bcl-2L1 (Bcl-X) mRNA forms two functionally distinct mRNAs. Bcl-XL is the longest isoform and acts as an inhibitor of apoptosis. On the other hand, Bcl-XS is the shortest isoform, which binds and inhibits Bcl-2 and then inhibits survival. Bcl-XL directly binds and closes the VDAC mitochondrial channel where BAX and BAK allow cytochrome c transition to cytosol (23).

Irregular CDK activity is frequently observed in malignant melanoma cells; thus, CDK inhibition is a rational approach to lead cell cycle arrest and then apoptosis (24,25). Flavopiridol is a semisynthetic flavanoid obtained from *Dysoxylum binectariferum* (26). Flavopiridol is reported to inhibit *in vitro* cell growth through CDKs (CDK2, CDK4, CDK6) in G1/S or G2/M of the cell cycle (27,28). There have also been several studies showing flavopiridol's apoptotic effects on melanoma cell lines (29). Combination studies of the flavopiridol and platin groups or other chemotherapy agents also show preclinical and clinical effects in terms of inhibiting the cell cycle and triggering apoptosis (30-32).

Proliferating cell nuclear antigen (PCNA) is a sliding clamp of DNA polymerase delta which catalyze DNA synthesis and take part in DNA repair (33). Thus, it is important to find PCNA decrease in order to get antiproliferative results of an applied agent.

The therapeutic advantage of DNA damaging agents relies on their ability to inhibit the cell cycle and enhance apoptosis in tumor tissue. It is thus important to evaluate the proliferative and apoptotic response against antineoplastic agents *in vitro* and *in vivo*. In this study, we aimed to examine the antiproliferative effects of the flavopiridol and oxaliplatin combination on the p16INK4A deficient melanoma cell line B16F10 and also its apoptotic effects on an allograft tumor model as a single agent.

## MATERIALS AND METHODS

### Cell lines and cell culture

The murine melanoma cell line B16F10 was obtained from American Type Culture Collection. Cells were maintained in the Dulbecco's modified Eagle (DMEM) medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin (all from Hyclone, USA). Cells were grown in a humidified incubator containing 5% CO<sub>2</sub> and 95% air at 37°C. B16F10 cells do not express p16INK4A and p14ARF genes.

### Reagents

Flavopiridol was obtained from Enzo (USA). A 10 mg/mL stock solution was prepared in dimethyl sulfoxide (DMSO). Oxaliplatin was purchased from Tocris (USA). A 86.33 mg/mL stock solution was prepared in DMSO.

### Animal studies

Eight weeks old C57BL6 mice weighing 25 g were purchased from the Laboratory of Animal Breeding and Experimental Research Center of Gazi University. Local Institutional Committee for the Ethical Use of Animals of Gazi University approved experimental procedures. Animals were fed with standard diet and water. C57BL6 mice received an injection of 5×10<sup>6</sup> B16F10 cells in 200 µL phosphate-buffered saline (PBS) subcutaneously. Twenty-four C57BL6 mice bearing B16F10 tumors were separated into four groups, six mice per group. Groups received intraperitoneal injections of DMSO, 2.5 mg/kg, 5.0 mg/kg and 10.0 mg/kg of flavopiridol at day 12 and 15 after tumor implantation. Mice from the control and flavopiridol groups were sacrificed by cervical dislocation after the last day of drug application. Tumor tissues were retrieved for analysis.

### Cell viability

Cell viability was determined by MTT assay. Cells were seeded in DMEM at 7×10<sup>3</sup> cells/200 µL per well in 96 well plates for 24h at 37°C. When cells were attached after 24 h, cells were treated with DMSO (control group), only flavopiridol (50 nM; 100 nM; 200 nM; 400 nM), only oxaliplatin (40 µM; 80 µM) and a combination of flavopiridol and oxaliplatin for 24 and 48 hours. After incubation time, a 10 µL MTT solution (0.5 mg/mL) was added to each well. After 4h of MTT incubation at 37°C, a 100 µL crystal dissolving buffer was added and the plates were gently shaken on an orbital shaker for 5 min. The absorbance at 570 nm was measured with a microplate reader. Each treat-

ment was repeated four times. The mean absorbance of four wells was used as an indicator of relative cell growth.

### RNA extraction and cDNA synthesis

3×10<sup>6</sup> B16F10 cells were seeded in 6 well plates and incubated with DMSO (control group) and flavopiridol (50 nM; 100 nM; 200 nM) for 24 and 48 hours. B16F10 injected C57BL6 mice also received 2.5, 5.0 and 10.0 mg/kg flavopiridol and sacrificed. *In vitro* total RNA was extracted with the "High Pure RNA Isolation kit" (Roche, Germany) protocol. *In vivo* total RNA was extracted from subcutaneous tumors (approximately 50-100 mg) using the Trizol reagent. The RNA-containing pellet was treated with 1-5 U RNase-free DNase per µg RNA and incubated at 37°C for 30 min before washing with 75% ethanol. The amount and quality of the RNA of each sample were determined by measuring the absorbance at 260 and 280 nm using the Nanodrop spectrophotometer (NanoDrop ND-1000, NanoDrop Technologies, USA). Total RNA (1 µg) was reverse transcribed in a 20-µL reaction mixture using random hexamers and the Transcriptor First-Strand cDNA Synthesis kit (Roche, Germany) according to manufacturer instructions. cDNA was used as a template for real time quantitative PCR analysis.

### Quantitative real-time polymerase chain reaction PCR (reverse transcription (RT)-PCR) analysis

BRAF and BCL2L1 mRNA expression levels were measured using RT-PCR with the LightCycler 480 (Roche, Germany). β-actin (ACTB) was used as a housekeeping gene in order to normalize BRAF and BCL2L1 expression levels. Probes and primers spanning exon-exon boundaries for each gene assay were designed using the Universal Probe Library (UPL). Primer and UPL probe numbers are presented in Table 1. The reaction mixture was prepared in 96 well plates containing 1 X LightCycler Taq-Man Master reaction mixture. Each sample was tested three times.

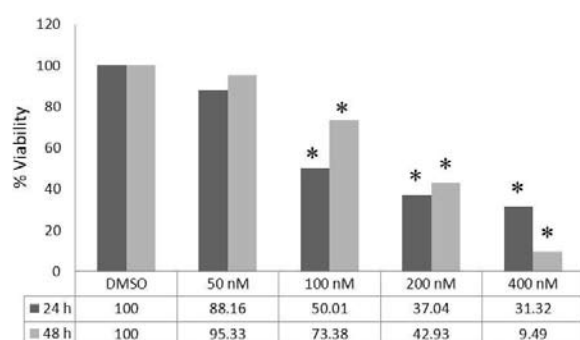
### Caspase 9 protein activity

2×10<sup>6</sup> cells were seeded in 6 well plates and incubated with DMSO (control group) and flavopiridol (50 nM and 100 nM) for 24 and 48 hours. B16F10 injected C57BL6 mice also received 2.5, 5.0, and 10.0 mg/kg flavopiridol and sacrificed the next day after the last injection. After sacrificing, tumors were stored at -80°C for protein activity assays. Protein concentration of each sample was determined using the BCA kit (Thermo, USA). Caspase 9 protein activity of each sample was measured by the "Caspase 9 Colorimetric

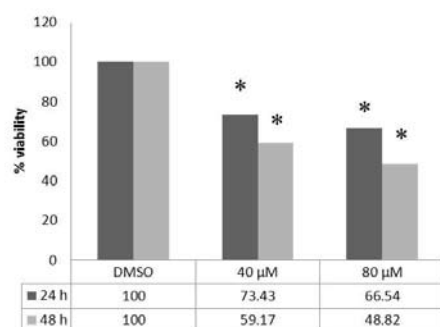
Assay Kit" (BioVision, Palo Alto, CA) according to the kit protocol.

### Caspase 3/7 protein activity

$2 \times 10^6$  cells were seeded in 6 well plates and incubated with DMSO (control group) and flavopiridol (50 nM and 100 nM) for 24 and 48 hours. B16F10 injected C57BL6 mice also received 2.5, 5.0, and 10.0 mg/kg flavopiridol and sacrificed the next day after the last injection. After sacrificing, tumors were stored at  $-80^\circ\text{C}$  for protein activity assays. Protein concentration of each sample was determined using the BCA kit (Thermo, USA). Caspase 3/7 protein activity was measured using the "Anaspec Sensolyte Homogeneous AFC Kaspaz 3/7 Assay Kit" (Anaspec, USA), according to the assay protocol.



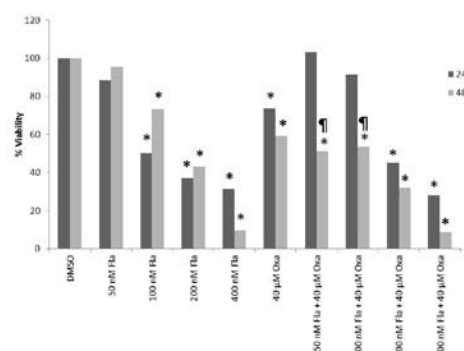
1. a)



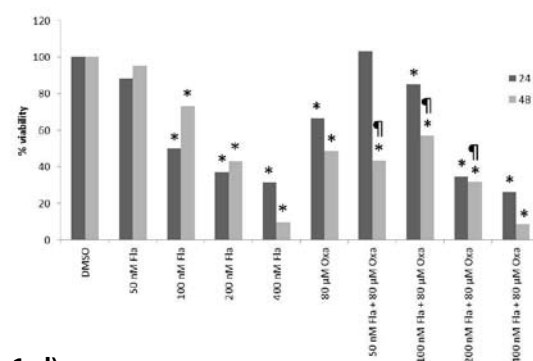
1. b)

### Immunohistochemistry

After the animals were sacrificed their tumors were excised, fixed in formalin, and embedded in paraffin for immunohistochemical staining. Paraffin sections were rehydrated in a series of xylene and ethanol washes. Antigen retrieval was carried out using a preheated target retrieval solution (pH 6.0) (Dako, Carpinteria, CA, USA). For immunohistochemistry, Vectastain Elite ABC kits (Vector Laboratories) were used according to manufacturer's instructions for blocking, dilution of primary antibody, and labeling. Sections were incubated with primary antibody against PCNA (Abcam, #500-2854) and Bcl-2 (Dako, #102230) overnight at  $4^\circ\text{C}$ . 3,3'-diaminobenzidine was prepared fresh from tablets (Sigma-Aldrich). Positive cells for each antibody of all mice from each group were counted using microscopy with estimation of intensity of staining ( $<10$  weak, 11-50% medium, and 51-80% strong).



1. c)



1. d)

**Figure 1. a)** The proliferation inhibitory effects of flavopiridol on B16F10 cells followed by the MTT assay. Viability rates of flavopiridol were shown in a time- and dose-dependent manner. B16F10 cells were treated with 50, 100, 200, and 400 nM of flavopiridol for 24 and 48 hours.  $*P < 0.05$

**b)** The proliferation inhibitory effects of oxaliplatin on B16F10 cells followed by the MTT assay. Viability rates of oxaliplatin were shown in a time- and dose-dependent manner. B16F10 cells were treated with 40 and 80  $\mu\text{M}$  of oxaliplatin for 24 and 48 hours.  $*P < 0.05$

**c)** The proliferation inhibitory effects of the flavopiridol and 40  $\mu\text{M}$  oxaliplatin combination on B16F10 cells followed by the MTT assay. Viability rates after flavopiridol and oxaliplatin administration were shown in a time- and dose-dependent manner.  $*P < 0.05$  Dimethyl sulfoxide (DMSO) versus treated group or combination;  $\pi P < 0.05$  Combination versus flavopiridol treated group.

**d)** The proliferation inhibitory effects of the flavopiridol and 80  $\mu\text{M}$  oxaliplatin combination on B16F10 cells followed by the MTT assay. Viability rates after flavopiridol and oxaliplatin administration were shown in a time- and dose-dependent manner.  $*P < 0.05$  DMSO versus treated group or combination;  $\pi P < 0.05$  Combination versus flavopiridol treated group.

**Table 1.** Primer sequences and probe numbers

Gene	Forward primer	Reverse primer	UPL Probe No.
ACTB	CTAAGGCCAACCGTGAAAAG	ACCAGAGGCATACAGGGACA	64
BRAF	GCTGGGACACGGACATT	GCAAAAGTCACAAAATGCTAAGG	55
BCL2L1	GTACCTGAACCGGCATCTG	GGGGCCATATAGTTCACAA	75

## Statistical analysis

Differences in cytotoxicity, immunohistochemical staining values, and protein activity levels were analyzed using SigmaStat software (version 12.0) by using the Student's t-test and one-way analysis of variance (ANOVA).  $P < 0.05$  were considered significant. Statistical significance of differences in BRAF and BCL2L1 mRNA expression levels was analyzed by the relative expression software tool (REST) designed for group-wise comparison and statistical analysis of relative expression results.

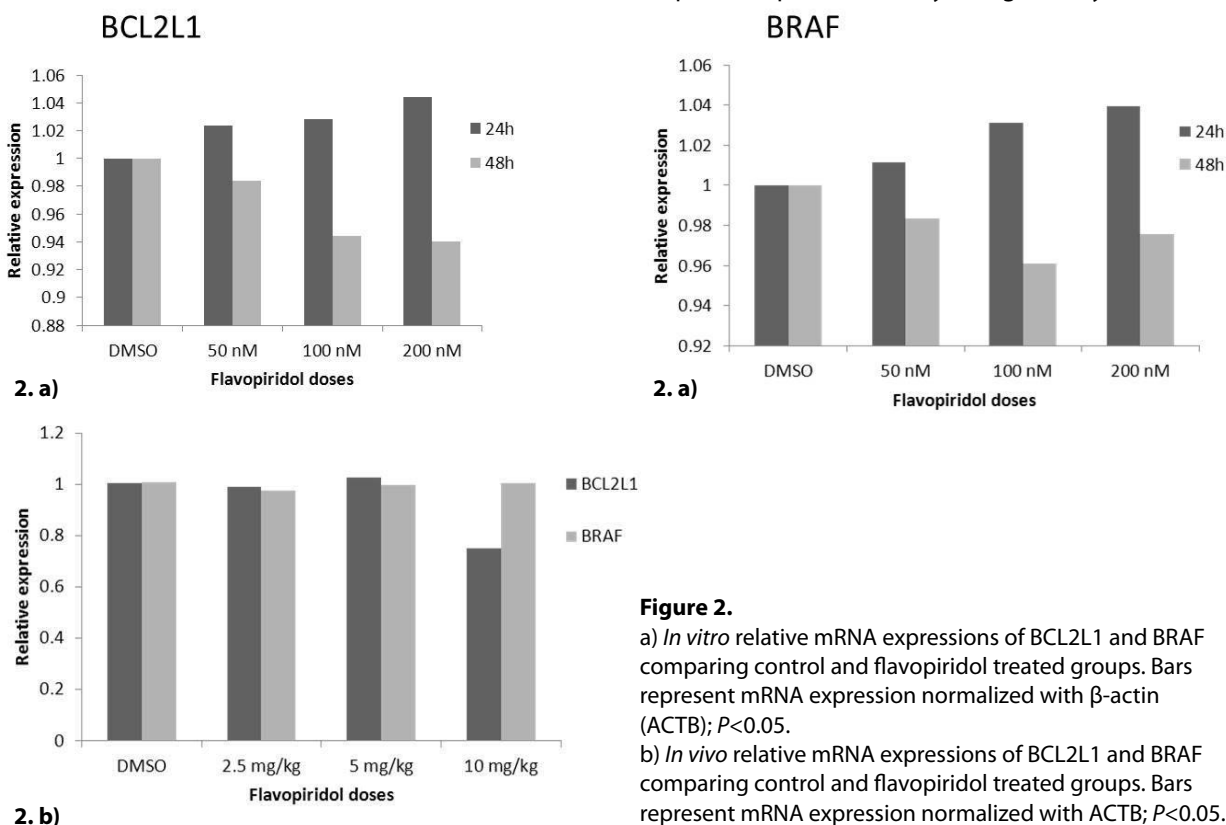
## RESULTS

B16F10 cell proliferation was significantly decreased at 100, 200, and 400 nM concentrations of flavopiridol at 24 and 48 hours.  $IC_{50}$  (Inhibition Concentration) value of flavopiridol was determined as 100 nM at 24 hour incubation (Figure 1, a). Oxaliplatin inhibited B16F10 cell proliferation at the concentrations of 40 and 80  $\mu$ M at 24 and 48 hours (Figure 1, b). However, the combination of flavopiridol and oxali-

platin did not have as much antiproliferative effects as single agents alone (Figure 1, c and d). After the ineffective combination results, we chose to apply flavopiridol as a single agent in all following experiments.

*In vivo* and *in vitro* BCL2L1 and BRAF relative mRNA expression levels did not differ significantly between the control and flavopiridol groups (Figure 2, a and b). Although *in vitro* BCL2L1 and BRAF mRNA expression levels were decreased with flavopiridol treatment, the difference was not statistically significant (Figure 2, a).

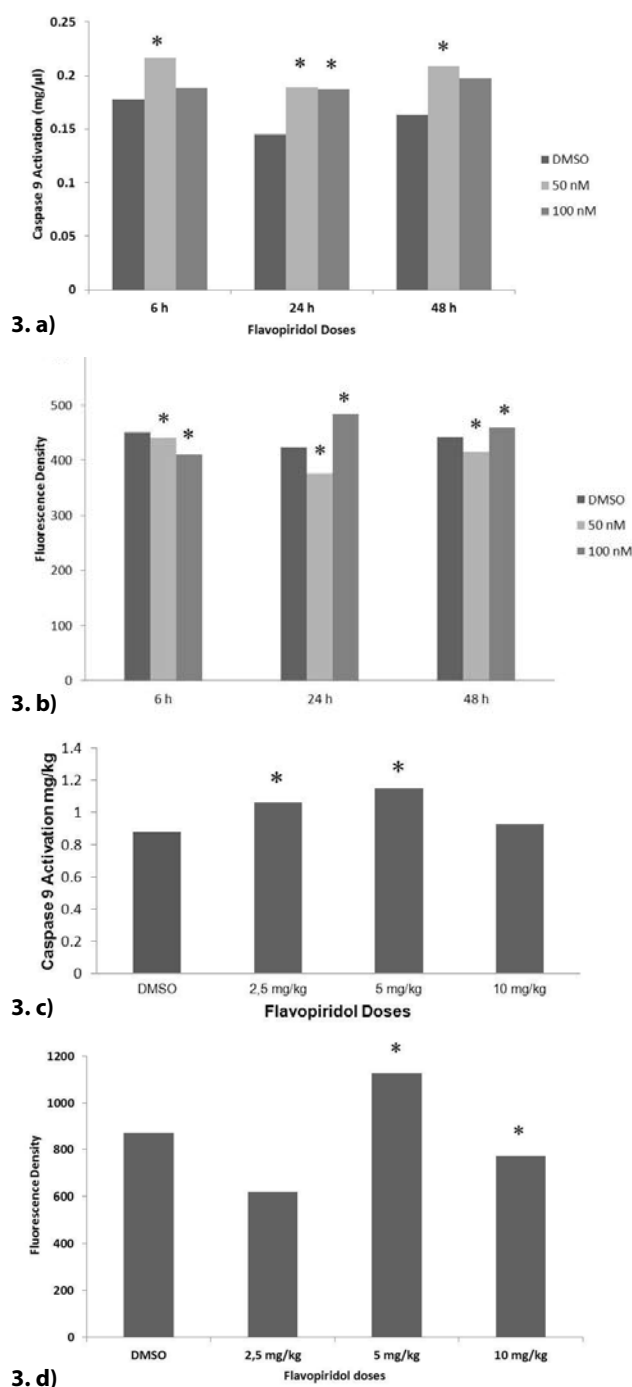
When B16F10 cells were treated with 50 and 100 nM of flavopiridol for 6, 24, and 48 hours, caspase 9 protein activity had a tendency to increase in all groups. Caspase 9 activity was significantly increased at 50 nM concentration at 6, 24, and 48 hours. Also, 100 nM of flavopiridol increased caspase 9 protein activity significantly at 24 hours (Figure 3, a). However, caspase 3/7 protein activity was generally decreased



**Figure 2.**

a) *In vitro* relative mRNA expressions of BCL2L1 and BRAF comparing control and flavopiridol treated groups. Bars represent mRNA expression normalized with  $\beta$ -actin (ACTB);  $P < 0.05$ .  
b) *In vivo* relative mRNA expressions of BCL2L1 and BRAF comparing control and flavopiridol treated groups. Bars represent mRNA expression normalized with ACTB;  $P < 0.05$ .





**Figure 3.** a) Increased *in vitro* caspase 9 protein activity in B16F10 cells in a time- and dose-dependent manner after flavopiridol administration. \* $P < 0.05$   
b) *In vitro* caspase 3/7 protein activity in B16F10 cells in a time- and dose-dependent manner after flavopiridol administration. \* $P < 0.05$   
c) Increased *in vivo* caspase 9 protein activity of B16F10 allograft tumors in C57BL6 mice after flavopiridol treatment. \* $P < 0.05$   
d) *In vivo* caspase 3/7 protein activity of B16F10 allograft tumors in C57BL6 mice after flavopiridol treatment. \* $P < 0.05$

at 50 nM flavopiridol concentration at all hours. On the other hand, 100 nM of flavopiridol increased caspase 3/7 activity significantly at 24 and 48 hours (Figure 3, b).

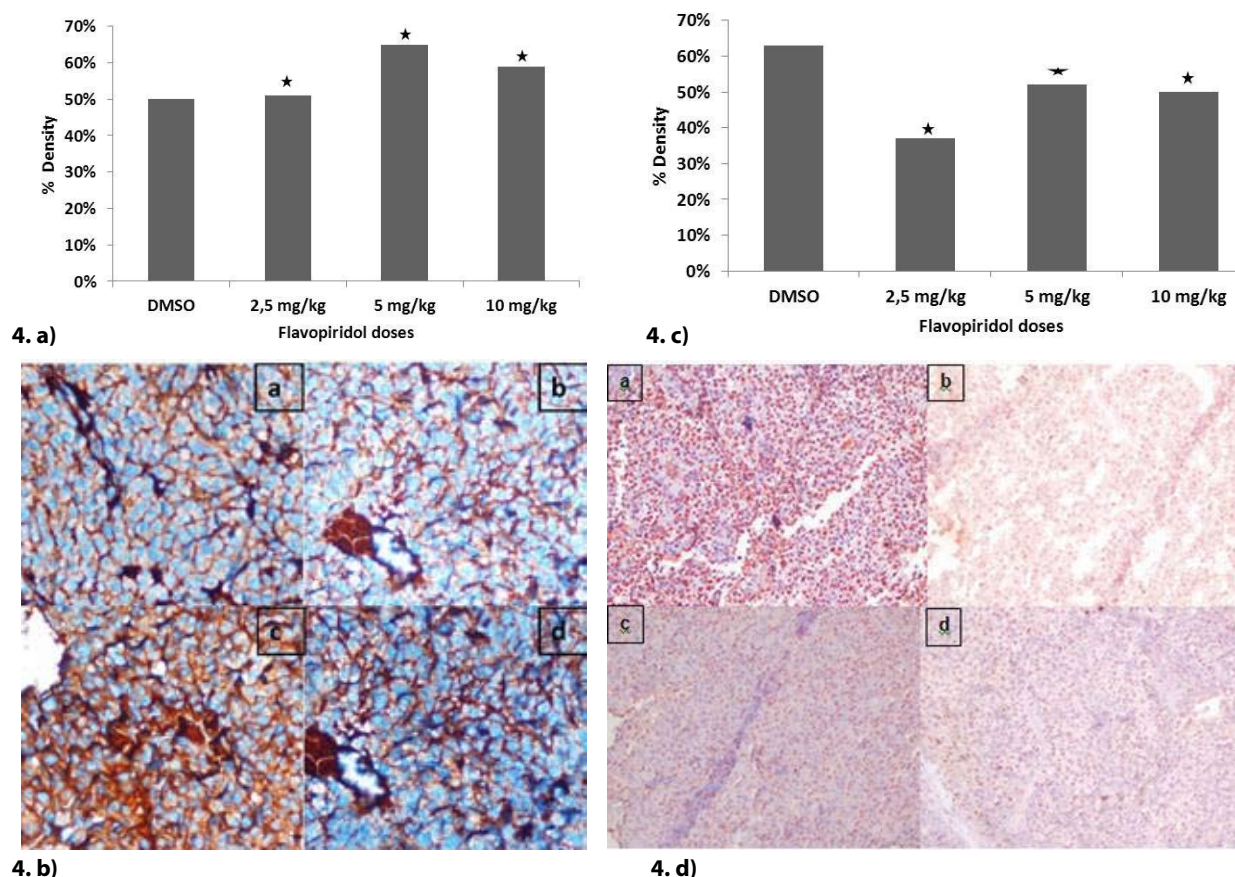
When C57BL6 mice were injected with B16F10 cells, tumors were formed after 7 days. Flavopiridol was applied two times intraperitoneally at the concentrations of 2.5, 5.0, and 10.0 mg/kg. All RNA and protein analysis was done from the same tissues. *In vivo* caspase 9 protein activity was significantly increased at 2.5 and 5.0 mg/kg of flavopiridol concentrations (Figure 3, c). At the same time, caspase 3/7 activity was significantly increased at 5.0 and 10.0 mg/kg flavopiridol (Figure 3, d).

Interestingly, we did not observe a decrease at Bcl-2 staining with flavopiridol treatment upon immunohistochemical analysis (Figure 4, a and b). Bcl-2 was significantly increased with flavopiridol treatment in each treatment group. However, PCNA levels significantly decreased in coordination with caspase levels in the flavopiridol treated groups (Figure 4, c and d). This data was also correlated with decreased *in vitro* proliferation rates.

## DISCUSSION

Malignant melanoma has 10-year survival rates less than 10% and has been difficult to treat for clinicians (1). However, several molecular approaches have helped understand the biology of malignant melanoma and lead to progress in treatment strategies. Driver BRAF and MEK gene mutations are the most famous molecular targets. To date, vemurafenib and dabrafenib (mutant BRAF inhibitors), (34) trametinib (MEK1/2 inhibitor) (35), and their combinations (36) have been used and approved by the FDA for metastatic malignant melanoma. However, resistance mechanisms such as the alternative pathway or CDK4/6 activations affect response rates even for combination therapies (37). The small molecule inhibitors those target the antiapoptotic BCL2 family members or IAPs are important to understand the apoptosis resistance mechanisms. Although melanoma cells are generally insensitive to these single agents, combination therapies have yielded promising results (38). CTLA-4, PD-1, and PDL-1 targets have also been tried in clinical trials (39,40) with promising results as combination therapies (41). Thus, even though new molecular targets and their inhibitors have been determined, blocking the cell cycle is still a rational option for inhibiting tumor cell proliferation.

Flavopiridol, the first approved CDK inhibitor, has been shown to mimic p16, which is frequently lost or mutated in malignant melanoma. In this study,



**Figure 4.** a) Quantification of immunohistochemistry for *in vivo* B-cell lymphoma 2 (BCL-2) protein expression of B16F10 allograft tumors in C57BL6 mice after flavopiridol treatment. Columns represent mean percentage of tumor cells with positive staining. \* $P < 0.05$

b) Representative images of immunohistochemistry for BCL-2 staining of B16F10 allograft tumors in C57BL6 mice after dimethyl sulfoxide (DMSO) (a), 2.5 mg/kg flavopiridol (b), 5.0 mg/kg flavopiridol (c), and 10.0 mg/kg flavopiridol treatment (×200)

c) Quantification of immunohistochemistry for *in vivo* proliferating cell nuclear antigen (PCNA) protein expression of B16F10 allograft tumors in C57BL6 mice after flavopiridol treatment. Columns represent mean percentage of tumor cells with positive staining. \* $P < 0.05$

d) Representative images of immunohistochemistry for PCNA staining of B16F10 allograft tumors in C57BL6 mice after DMSO (a), 2.5 mg/kg flavopiridol (b), 5.0 mg/kg flavopiridol (c), and 10.0 mg/kg flavopiridol (d) treatment (×200)

we investigated the effects of flavopiridol at various concentrations in anti-proliferative and apoptotic events and gene expression profiles in a p16 deficient B16F10 mouse melanoma cell line and an allograft tumor model in C57BL6 mice. In our study,  $IC_{50}$  value was determined as 100 nM for 24 hours in flavopiridol applied B16F10 cells. In agreement with our study, Robinson *et al.* reported that cell cycle was inhibited even for low doses of flavopiridol (12.5 nM – 25.0 nM) and caused apoptosis at 100 nM. Although higher concentrations decreased  $IC_{50}$  concentration they did not affect expression of anti-apoptotic BCL2 expression (29). Jackman *et al.* showed flavopiridol induced cell cycle arrest and apoptosis at lower concentra-

tions. However, reduced CDK activity was observed at higher flavopiridol doses in acute lymphoblastic leukemia cells (42).

The antiproliferative effect of flavopiridol has been shown to increase when applied with various agents in clinical and preclinical studies (30-32). In our study, oxaliplatin decreased proliferation at 80  $\mu$ M ( $IC_{50}$  concentration) at 48 hours. However, combination of flavopiridol and oxaliplatin did not have as much antiproliferative effects as single agents alone. In a phase I study, a flavopiridol and oxaliplatin/fluorouracil (5-FU)/folinic acid combination was safe and tolerable for advanced solid tumors (31).

The apoptosis mechanism triggered by flavopiri-

dol has still not been well-established. It has however been shown that it may be BCL2 dependent or independent. Ma *et al.* showed flavopiridol treatment can enhance apoptosis through stabilizing E2F1 and transcriptionally repressing MCL-1 in H1299 lung carcinoma cells (43). Lucas *et al.* also showed lower doses of flavopiridol increased apoptotic NOXA protein levels and sensitizes MM200 and Me4405 melanoma cells to ABT-737, a BH-3 mimetic agent in 2D and 3D cultures (44). According to our findings, BCL2L1 mRNA expression did not change significantly in control and flavopiridol treated groups in both *in vitro* and *in vivo* studies. We also did not observe any decrease in BCL2 protein levels in flavopiridol treated allograft tumors. In agreement with our study, it has been shown that flavopiridol induced apoptosis independently from BCL2 expression (45). On the other hand, flavopiridol has downregulated BCL2 mRNA and protein expression and induced apoptosis within 24 hours in B cell leukemia cell lines (46). It has been shown that BCL2 expression was induced by flavopiridol in leukemic blasts in adult acute leukemia patients (47). The studies examining flavopiridol's apoptotic effects do not clearly explain the role of BCL2 protein expression in apoptosis. Although Konig *et al.*, Sato *et al.*, and Newcomb *et al.* showed BCL2 decreased as a result of the apoptotic effects of flavopiridol in different cell lines, we could not find any difference between flavopiridol treated and untreated groups in our study (46,48,49). In line with our study, flavopiridol induced apoptosis through caspase 3 activation independently from BCL2 or p53 function in chronic lymphocytic leukemia cells (50).

It is also known that flavopiridol can induce apoptosis through caspase-dependent or independent mechanisms according to the mutational status and histologic type of the cell. In our study, flavopiridol induced caspase 9 activation both *in vivo* and *in vitro*. Also, caspase 3/7 was upregulated at higher doses of flavopiridol in the cells and tumor model. In glioma cell lines, flavopiridol did not activate caspase 3, poly ADP ribose polymerase (PARP), or caspase 8 independently from tumor suppressor pathway changes such as retinoblastoma and p53. Mitochondrial damage and cytochrome c release were also not observed in those cells (47). However, Li *et al.* showed flavopiridol caused the release of cytochrome c from mitochondria, activated caspase 9, 8, and 3, increased proapoptotic BAX, and decreased BCL2 protein levels in drug resistant osteosarcoma and Ewing's family tumor cells (51). Additionally, Puppo *et al.* determined that flavopiridol increased caspase 3 activation, however it had no effect in stimulating caspase 9 and led to apoptosis through mitochondrial pathway activation

in neuroblastoma cells (52). G2 arrest and cyclin B1 downregulation were also observed in low concentrations of flavopiridol application in rhabdoid cells. When flavopiridol was combined with 4OH-tamoxifen it did not affect flavopiridol induced G2 arrest but led to caspase-3/7 activation. p53 inhibition by siRNA removed flavopiridol-induced G2 arrest but enhanced apoptosis by activating caspase 2 and 3 (53).

On the other hand, Mahoney *et al.* have shown that flavopiridol protects chronic lymphocytic leukemia cells from autophagy, which causes resistance to cyclin-dependent kinase targeted therapies [(54,55)]. However, Xiao *et al.* showed a HSP90 inhibitor 17-AAG can sensitize mantle cell lymphoma cells to flavopiridol induced autophagy and also enhanced apoptosis (56). Immunochemical evaluation of PCNA protein expression is one of the methods used to determine cellular proliferation level in tumor tissue. PCNA is synthesized in the S phase of cell cycle that is an indicator of DNA synthesis; it is important in determining mitosis number and mitotic index (57). Verdager *et al.* observed decreased PCNA expression and apoptosis after flavopiridol application in primary cultures of rat cerebellar granule cells (58). In our *in vivo* study, the decrease in PCNA protein expression and increase in caspase 3/7 and caspase 9 activities may show that flavopiridol stopped the cell cycle and led to apoptosis through the mitochondrial pathway.

## CONCLUSION

Apoptosis was induced by flavopiridol independently from BCL2L1 through the mitochondrial pathway in which caspase 3/7 and caspase 9 activated in p16INK4A and p14ARF mutant cell line B16F10 and B16F10 injected C57BL6 mice. PCNA expression was decreased by flavopiridol, which caused cell cycle arrest in the melanoma model. Nonetheless, while there are still many mechanisms and unclear results to investigate, flavopiridol can be used as a cell cycle inhibitor and apoptosis inducer in malignant melanoma.

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